EBV Viral Load in Tumor Cells Did Not Predict Tumor Extensiveness in Nasopharyngeal Cancer

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Abstract

Background:

Nasopharyngeal cancer is commonly associated with EBV infection, especially the undifferentiated non keratinized histology. EBV DNA quantification through nasopharyngeal brushing was previously reported not related with disease stages. This study aimed to reinvestigate relationship of EBV viral load in tumor tissue with tumor extensiveness by more accurate EBV DNA quantification through microscopically confirmed tumor cells from nasopharyngeal biopsy.

Method:

The specimens for EBV DNA quantification was derived from histopathology slides which was pre-treated following QIAsymphony® SP Protocol for tissue DNA extraction. Then the extracted DNA underwent real time Polymerase Chain Reaction (RT-PCR) using artus® EBV RG PCR Kit for EBV DNA quantification. The tumor volume was determined by delineating gross tumor based on 3D imaging of the patient's nasopharynx.

Result:

Twenty-four subjects were included in this study. All subjects were stage III and above with more males (75%) than females. EBV viral load in tumor cells were found to have no correlation with tumor volume both in local and nodal. The median local tumor volume was 81.3 cm3 ± 80 cm^3. The median EBV viral load in tumor cells was 95,644.8 copies/100ng of DNA ± 224,758.4 copies/100ng of DNA. The median nodal or regional tumor volume was 35.7 cm3 ± 73.63 cm^3.

Conclusion:

EBV viral load from tumor cells from nasopharyngeal biopsy has no relationship with tumor extensiveness in nasopharyngeal cancer. The presence and number of EBV in tumor cells did not translate into larger or smaller tumor. The EBV viral proteins and RNAs were perhaps more likely to confer some prognostic information due to the facts that those molecules were related with carcinogenesis.

Introduction

Development of nasopharyngeal cancer is very much related with Epstein Barr Virus (EBV).[1] EBV was found in approximately all nasopharyngeal cancer with undifferentiated non keratinized histology.[2,3] EBV infection could become dormant within host epithelial cells.[4] This EBV in its dormant or latent phase would express various viral molecules that have been shown to be responsible for inducing carcinogenesis in nasopharyngeal cancer.[5–8]

Due to a tight association between EBV and nasopharyngeal cancer, EBV viral load have been proposed to be a biomarker that could possibly serve as a screening tool, guide treatment or inform prognosis. Detectable EBV viral load in plasma have been shown to be able to inform asymptomatic patient for greater risk of developing nasopharyngeal cancer.[9] Furthermore, EBV viral load in plasma was found to be prognostic especially in predicting risk of distant recurrence.[10] However, EBV viral load from local nasopharyngeal specimens have not been shown to be prognostic.[11]

The EBV genotype from circulating plasma was found to be similar with the EBV genotype from tumor cells.[12] That finding indicating that EBV detected from plasma was very likely to be derived from EBV in tumor cells from the nasopharynx. Therefore, with the knowledge suggesting a prognostic role of plasma EBV viral load, the EBV DNA from tumor cells in nasopharynx was thought to be able to confer an even stronger prognostic value.

The previous study that found negative relationship between EBV virus from tumor cells and tumor burden or disease stage was thought due to the specimen collected from nasopharyngeal brushing might not represent the true EBV samples from tumor cells.[11] Therefore, a more accurate and representative design with EBV samples collected directly from microscopically confirmed tumor cells were used in this study to seek the role of EBV viral load in tumor cells.

Method

Study Design and Subject Recruitment

This study recruited subjects with microscopically confirmed nasopharyngeal carcinoma from nasopharynx biopsy. All subjects were 18 years and above. They all consented to be included in this study. All subjects underwent 3D imaging with either CT Scan or MRI for nasopharynx to assess initial pre-treatment tumor extension. The pre-treatment imaging utilized in this study was within 1 month of nasopharynx biopsy.

Sample Processing For Ebv Dna Quantification

The specimens were derived from a paraffin embedded tissue of those confirmed nasopharyngeal cancer patients. A 4 μm cut thickness of those paraffin embedded tissue were put into a slide. The slide then underwent standard hematoxylin-eosin staining. The stained slides were reviewed and the exact location of tumor cells were marked initially with a permanent marker by a senior pathologist. The marked slides were then re-marked again by carving the glass slides with a diamond pen. The permanently carved slides underwent pre-treatment for deparaffinization using xylene. The pre-treatment process of the slides was following QIAsymphony® SP Protocol for tissue.

After pre-treatment, the DNA from the specimens was extracted and purified using QIAsymphony DSP DNA Mini Kit. The DNA extracted was then quantified with a spectrophotometer following manufacturer

guideline. The DNA specimens were then diluted until reaching 100 ng DNA per ml of solution. Those homogenous DNA content from all the specimens were used for real time Polymerase Chain Reaction (RT-PCR) using artus® EBV RG PCR Kit for EBV DNA quantification.

Tumor Volume Determination

Tumor extensiveness in this study was referred to tumor volume. The 3D imaging done within 1 month of nasopharyngeal biopsy for all subjects were used for the basis of tumor volume determination. The tumor volume was determined by contouring the gross tumor locally in nasopharynx and regionally in cervical lymph nodes. The contouring was done using Eclipse™ software. The contouring was structured separately for gross tumor on local and regional to obtain local tumor volume and nodal tumor volume. The contours were performed by 2 radiation oncologists, independently. The final contours structures were discussed together to reach a consensus to determine the final structures. The volume for each structures were then recorded.

Statistical Analysis

A correlation test using Pearson's Correlation Test was performed between tumor volume and EBV viral load in tumor cells. The distribution for each numeric variable was determined using histogram. For the skewed distribution, if there was no correlation found, the data was re-arranged into logarithmic scale to obtain a normally distributed data. It was done whenever it was sensible and the basis of the data permit for conversion into logarithmic scale. A re-analysis of the correlation using Pearson's Correlation Test was done after normalization of data distribution to ascertain the presence or absence of the correlation.

Result

Subject Characteristics

Twenty-four subjects were included in this study. There was almost equal number of subjects under and up to age 50 and below 50. There were more males (75%) compared to females. All subjects were in locally advanced to advanced stage (stage III and above). There were 33.3% of subjects in advanced metastatic stage IVB. (see Table 1)

Table 1
Subject Characteristics

Characteristics	Number of Subjects (%)
Age	
≤ 50 years old	13 (54.2%)
> 50 years old	11 (45.8%)
Gender	
Male	18 (75%)
Female	6 (25%)
Stage*	
III	4 (16.7%)
IVA	12 (50%)
IVB	8 (33.3%)
Total	24 (100%)
* Based on AJCC Staging 8th Edition	

Relationship between Tumor Volume and EBV Viral Load in Tumor Cells

There was no correlation found between gross tumor volume at local nasopharynx with EBV viral load in tumor cells (see Fig. 1a). The median local tumor volume was 81.3 cm3 ± 80 cm^3. The median EBV viral load in tumor cells was 95,644.8 copies/100 ng of DNA ± 224,758.4 copies/100 ng of DNA. Higher EBV viral load in tumor cells at local nasopharynx did not result in larger tumor at local nasopharynx.

There was also no correlation found between gross tumor volume at nodal or regional nasopharynx with EBV viral load in tumor cells (see Fig. 1b). The median nodal or regional tumor volume was 35.7 cm3 ± 73.63 cm^3. Higher EBV viral load in tumor cells at local nasopharynx did not result in larger nor smaller tumor at nodal or regional nasopharynx.

The replication of EBV was following a geometric order, therefore the data distribution of EBV viral load was skewed if linear scale was used (see Fig. 2a). A converted scale from linear to logarithmic for EBV viral load in tumor cells have resulted in those data presented as normal distribution (see Fig. 2b). A re-analysis with EBV viral load in logarithmic scale was done.

There was also no correlation existed between both local tumor volume and nodal tumor volume with Log of EBV viral load in tumor cells from local nasopharynx (See Fig. 1c and 1d). The mean Log of EBV viral load in tumor cells from local nasopharynx was 4.94 Log copies/100 ng of DNA ± 0.64 Log copies/100 ng of DNA.

Discussion

EBV viral load in tumor tissue from nasopharyngeal biopsy has been shown in our study not related to baseline tumor extensiveness. Even though EBV is presence in almost all nasopharyngeal cancer, the number of virus presence within the cancer cells did not translate into greater or smaller tumor sizes. This finding indicating that EBV virus replication within the host cells does not affect cancer phenotype.

There are evidence suggesting that expression of several EBV viral oncogenic genes are related to cancer development.[4,13] These oncogenic proteins are probably the major driver of carcinogenesis, especially the proteins expressed by the latent EBV genes.[4] In acute EBV infection, the lytic phase will ensue with expression of lytic genes. After lytic infection the virus will reside and become dormant within the epithelial or lymphocytes by expression of various latent genes. This latent genes such as Latent Membrane Protein 1 (LMP1) and Epstein-Barr nuclear antigen 1 (EBNA-1) expressed in latent phase of EBV infection were found to contribute to cancer development.[4]

The viral protein LMP1 was associated with inactivation of several important genes such as tumor suppressor genes within the host cells.[4,14,15] The LMP1 inactivated tumor suppressor genes for instance Phosphatase and tensin homolog (PTEN) gene through the action of DNA methyltransferase 3b (DNMT3b).[15] This DNMT3b resulted in PTEN CpG island methylation.[15] Furthermore, the LMP1 expressed by EBV has also been shown to be able to methylate CDH-1 promoter region which eventually silencing E-cadherin gene. The cadherin is cell adhesion molecule in which deficient in this protein will promote cancer cells to easily metastasize.[16]

The EBNA-1 viral protein was associated with gene methylation process and genomic instability within the host genome.[4,14] The gene profiling study has shown that EBNA-1 could bound to promoter sites of many host genes and resulted in upregulation and downregulation of specific genes.[17] The presence of EBNA-1 was also related with elevated production of reactive oxygen species (ROS) within the host cells.[18] This elevated ROS further increased the likelihood of genomic instability and DNA damage.[18] In pre-clinical model, EBNA-1 expression was shown to be strongly associated with tumor growth.[19] All these findings indicating that EBNA-1 is required at least in part for tumorigenesis.

Another EBV molecule that is also highly transcribed in latent EBV infection was Epstein-Barr Virus - encoded RNAs (EBER). The EBER is a small segment of RNA molecule that is not expressed into protein, but it plays a major role in facilitating the process of immune escape.[4] The EBER can interfere with interferon stimulating gene, thereby blunting the immune response.[20] In concert with other EBV viral

proteins such as LMP1, the EBV could stimulate recruitment and activation of Treg into tumor microenvironment, therefore stimulating an even more permissive microenvironment for tumor growth.[21]

Based on all the findings above and the result of our study, the expression of these oncogenic viral genes is probably more likely to correlate with tumor progression rather than presence and the number of EBV virus alone. EBV viral load quantification in tumor tissue was probably useful to assist doubtful diagnosis of nasopharyngeal cancer, especially the endemic type with WHO type 3 histopathology.[22] Quantification EBV DNA in tumor cells might not be able to confer valuable prognostic information. However, quantification of viral proteins or RNAs might be able to provide some prognostic clue.

Conclusion

EBV viral load in tumor tissue from nasopharyngeal biopsy did not correlate with baseline pre-treatment tumor extensiveness. The presence and number of virus in tumor cells was probably not much informative, except for confirming diagnosis of nasopharyngeal carcinoma. EBV viral molecules such as LMP1, EBNA-1, and EBER were probably more likely to confer prognostic information.

Declarations

Ethics approval and consent to participate

This study has obtained ethical approval by Board of Ethic Committee from Faculty of Medicine, Universitas Indonesia on 11 March 2019 with ethical approval number KET-229/UN2.F1/ETIK/PPM.00.02/2019. All subjects consented prior to their enrollment to this study.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors have equal contributions. All authors read and approved the final manuscript.

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Conflict of Interest

None

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Figures

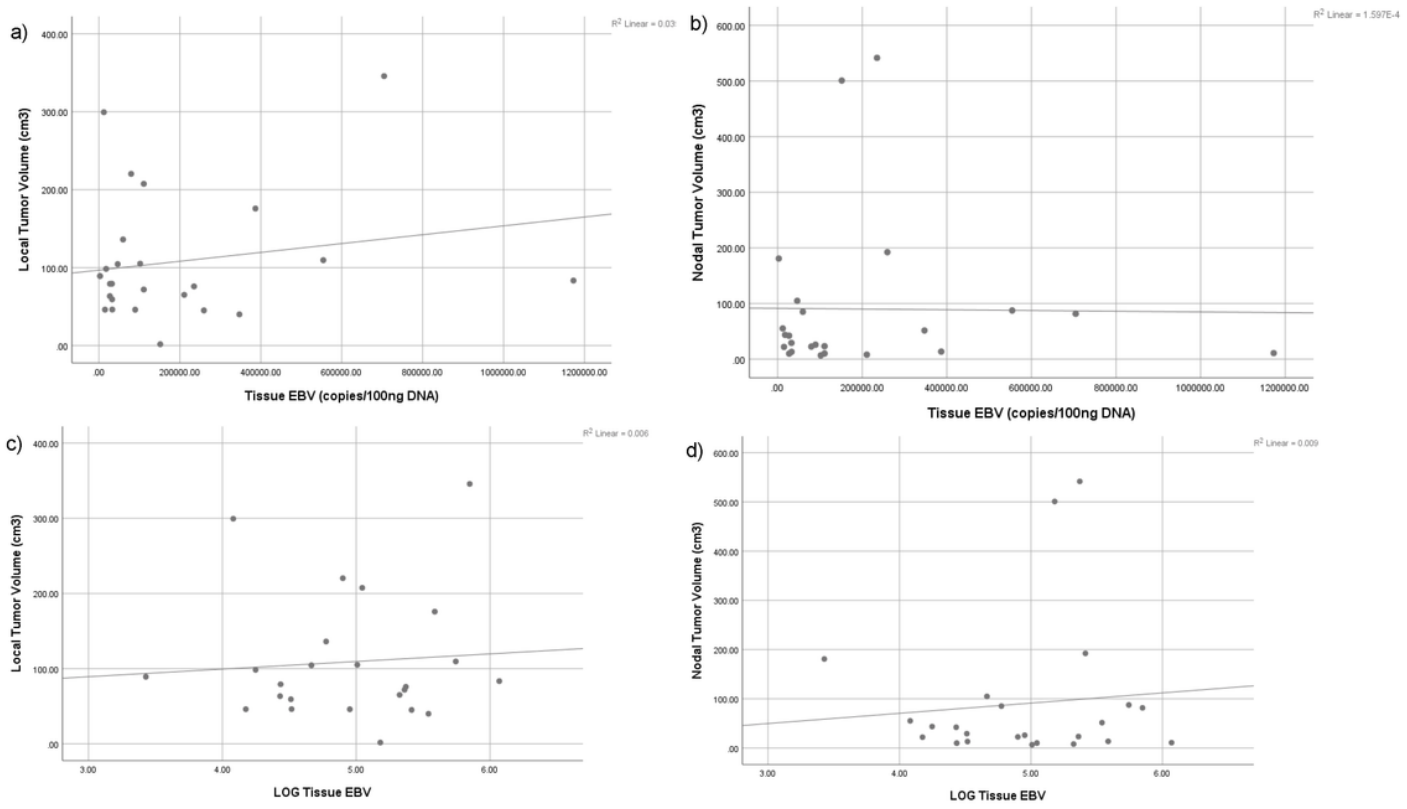

Figure 1: Scatterplot showing no correlation between a-b) EBV viral load in nasopharyngeal tumor tissue with a) local tumor volume, b) nodal tumor volume. c-d) Re-analysis of correlation using normalized distribution in logarithmic scale of EBV viral load in nasopharyngeal tumor tissue also showing no correlation with c) local tumor volume, d) nodal tumor volume

Figure 1

Scatterplot showing no correlation between a-b) EBV viral load in nasopharyngeal tumor tissue with a) local tumor volume, b) nodal tumor volume. c-d) Re-analysis of correlation using normalized distribution in logarithmic scale of EBV viral load in nasopharyngeal tumor tissue also showing no correlation with c) local tumor volume, d) nodal tumor volume

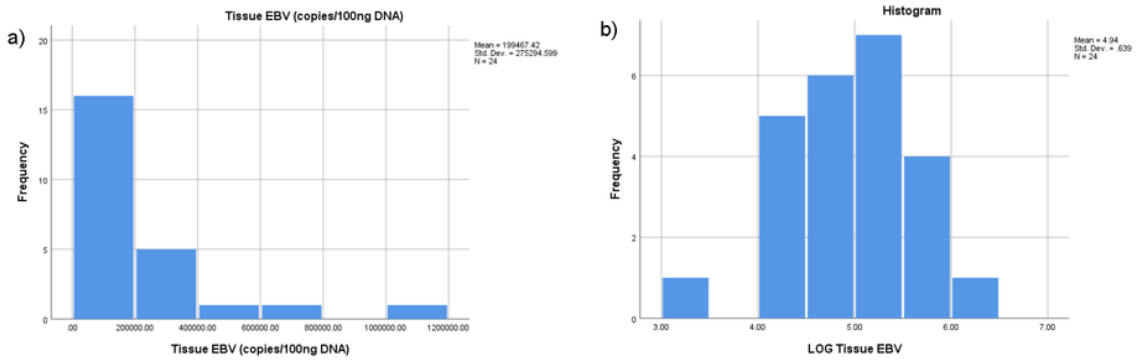

Figure 2: Histogram showing distribution of data for EBV viral load in tumor tissue from nasopharyngeal biopsy a) non-normal distribution with linear scale, b) normal distribution with logarithmic scale

Figure 2

Histogram showing distribution of data for EBV viral load in tumor tissue from nasopharyngeal biopsy a) non-normal distribution with linear scale, b) normal distribution with logarithmic scale